

Colchicine attenuates inflammatory cell infiltration and extracellular matrix accumulation in diabetic nephropathy

Jin Ji Li,^{1,2*} Sun Ha Lee,^{1*} Dong Ki Kim,^{1*} Ri Jin,² Dong-Sub Jung,¹ Seung-Jae Kwak,¹ Seung Hye Kim,¹ Seung Hyeok Han,¹ Jung Eun Lee,¹ Sung Jin Moon,¹ Dong-Ryeol Ryu,³ Tae-Hyun Yoo,¹ Dae Suk Han,¹ and Shin-Wook Kang¹

¹Department of Internal Medicine, College of Medicine, Brain Korea 21 for Medical Science, Yonsei University, Seoul;

²Nephrology and Dialysis Unit, Department of Internal Medicine, The Affiliated Hospital, Yanbian University Medical

College, JiLin, China; and ³Department of Internal Medicine, College of Medicine, Ewha Womans University, Seoul, Korea

Submitted 29 October 2008; accepted in final form 9 April 2009

Li JJ, Lee SH, Kim DK, Jin R, Jung D, Kwak S, Kim SH, Han SH, Lee JE, Moon SJ, Ryu D, Yoo T, Han DS, Kang S. Colchicine attenuates inflammatory cell infiltration and extracellular matrix accumulation in diabetic nephropathy. *Am J Physiol Renal Physiol* 297: F200–F209, 2009. First published April 15, 2009; doi:10.1152/ajprenal.90649.2008.—Recent studies have demonstrated that an inflammatory mechanism contributes to the pathogenesis of diabetic nephropathy (DN). It is also known that colchicine (Col) can prevent various renal injuries via its anti-inflammatory action. However, the effect of colchicine on DN has never been explored. This study was undertaken to elucidate the effect of colchicine on inflammation and extracellular matrix accumulation in DN. In vivo, 64 rats were injected with diluent (C; $n = 32$) or streptozotocin intraperitoneally (DM, $n = 32$). Sixteen rats from each group were treated with Col. In vitro, rat mesangial cells and NRK-52E cells were cultured in media with 5.6 mM glucose (NG) or 30 mM glucose (HG) with or without 10^{-8} M Col. Monocyte chemotactic protein-1 (MCP-1) mRNA expression was determined by real-time PCR (RT-PCR), and the levels of MCP-1 in renal tissue and culture media were measured by ELISA. RT-PCR and Western blotting were also performed for intercellular adhesion molecule-1 (ICAM-1) and fibronectin (FN) mRNA and protein expression, respectively, and immunohistochemical staining (IHC) for ICAM-1, FN, and ED-1 with renal tissue. Twenty-four-hour urinary albumin excretion at 6 wk and 3 mo were significantly higher in DM compared with C rats ($P < 0.05$), and colchicine treatment significantly reduced albuminuria in DM rats ($P < 0.05$). Col significantly inhibited the increase in MCP-1 mRNA expression and protein levels under diabetic conditions both in vivo and in vitro. ICAM-1 and FN expression showed a similar pattern to the expression of MCP-1. IHC revealed that the number of ED-1(+) cells were significantly higher in DM compared with C kidney ($P < 0.005$), and this increase was significantly attenuated by Col treatment ($P < 0.01$). In conclusion, Col prevents not only inflammatory cell infiltration via inhibition of enhanced MCP-1 and ICAM-1 expression but also ECM accumulation in DN. These findings provide a new perspective on the renoprotective effects of Col in DN.

inflammation; MCP-1; ICAM-1; fibronectin; afferent arteriole; contraction; losartan

INFILTRATION OF INFLAMMATORY cells in renal tubulointerstitium is commonly seen in both human diabetic patients and experimental diabetic animals (4, 12, 46). Monocytes/macrophages are the principle inflammatory cells found in the diabetic kidney, and recent studies have suggested that monocytes/macrophages play

an important role in the pathogenesis of glomerulopathy and tubulointerstitial lesions in diabetic nephropathy. The administration of anti-inflammatory agents, such as mycophenolate mofetil (MMF) (43) and retinoic acid (16), and the use of irradiation (40) reduced inflammatory cell infiltration and prevented renal injury in experimental diabetic animals. These findings suggest that an inflammatory mechanism may also contribute to the pathogenesis of diabetic nephropathy.

Monocytes/macrophages are extravasculated from the bloodstream and attracted to the target tissue through a process mediated by various chemokines and adhesion molecules such as monocyte chemotactic protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1. In the kidney, MCP-1 is expressed in mesangial cells and tubular epithelial cells (34, 39) and is known to be involved in the pathogenesis of various renal diseases, including diabetic nephropathy. Previous studies have demonstrated that plasma MCP-1 levels are increased in type 1 diabetes with microalbuminuria (3) and that urinary levels of MCP-1 are also increased in accordance with the extent of albuminuria (1, 28). Renal expression of ICAM-1, a cell surface glycoprotein that plays a major role in the regulation of interactions with immune cells and whose expression is upregulated at the sites of inflammation, is also known to be increased in experimental type 1 and type 2 diabetic animals (8, 42). These findings suggest that MCP-1 and ICAM-1 may play an important role in the pathogenesis of diabetic nephropathy via inducing inflammatory cell infiltration.

Colchicine is an “old” drug commonly used to relieve pain in acute gout and is known to inhibit the function and motility of granulocytes and other motile cells by binding to microtubular protein, which leads to the depolarization and disappearance of the fibrillary microtubular structures (17). In addition, colchicine has been used to prevent amyloidosis secondary to familial Mediterranean fever (31) and has been demonstrated to prevent experimental pulmonary and hepatic fibrosis by suppressing the release of fibronectin and stimulating tissue collagenase activity (21, 26, 36, 38). Furthermore, recent studies have shown that colchicine prevents renal injury in an animal model of chronic cyclosporine nephrotoxicity via its antiapoptotic and anti-inflammatory action (11, 24, 25). To our knowledge, however, there has not been a study that analyzed the effect of colchicine in diabetic nephropathy. In this study, we investigated the effect of colchicine on the expression of MCP-1, ICAM-1, and fibronectin in experimental diabetic kidney and in high glucose-stimulated mesangial cells and tubular epithelial cells. In addition, the relationship between

* J. J. Li, S. H. Lee, and D. K. Kim contributed equally to this work.

Address for reprint requests and other correspondence: S.-W. Kang, Yonsei Univ., College of Medicine, Dept. of Internal Medicine, 134 Shinchon-Dong, Seodaemun-Gu, Seoul, Korea 120-752 (e-mail: kswkidney@yumc.yonsei.ac.kr).

the extent of inflammatory cell infiltration and fibronectin expression in renal tissue was elucidated.

MATERIALS AND METHODS

Animals

All animal studies were conducted under an approved protocol. Rats weighing 250–280 g were injected with either diluent [$n = 32$, control (C)] or 65 mg/kg streptozotocin (STZ) intraperitoneally [$n = 32$, diabetes (DM)]. Diabetes was confirmed by tail-vein blood glucose levels on day 3 postinjection. After confirmation of diabetes, 16 rats from each group were treated with $30 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of colchicine intraperitoneally (C+Col, DM+Col). Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the study period. Eight rats from each group were killed after 6 wk and the remainders after 3 mo.

Body weights were checked biweekly, and kidney weights were measured at the time of death. Serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels, and 24-h urinary albumin were also measured at the time of death. Blood glucose was measured with a glucometer and 24-h urinary albumin excretion by ELISA (Nephra II, Exocell, Philadelphia, PA). Serum ALT, AST, BUN, and creatinine levels were determined by an automatic biochemical analyzer.

Cell Culture

Primary culture of mesangial cells was done as previously described (18). Identification of mesangial cells was performed by their characteristic stellate appearance in culture and confirmed by immunofluorescent microscopy for the presence of actin, myosin, and Thy-1 antigen and the absence of factor VIII and cytokeratin (Synbiotics, San Diego, CA). Mesangial cells and NRK-52E cells, immortalized rat tubular epithelial cells, were maintained in RPMI 1640 and DMEM medium, respectively, supplemented with 5% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 26 mM NaHCO_3 , and were grown at 37°C in humidified 5% CO_2 in air. Subconfluent mesangial cells and NRK-52E cells were serum restricted for 24 h, after which the medium was replaced by serum-free medium containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), or 30 mM glucose (HG) with or without 10^{-8} M colchicine (Col). At 24 h after the media change, cells were harvested and the conditioned culture media were collected.

Methylthiazolotetrazolium Assay

To examine the cytotoxicity of colchicine, mesangial cells and NRK-52E cells were cultured in 96-well culture plates, phenol red-free DMEM with 1 mg/ml of methylthiazolotetrazolium (MTT) was added to each well after the experimental periods and incubated for 2 h at 37°C in humidified 5% CO_2 in air. Extraction buffer (20% SDS,

50% N,N -dimethylformamide, pH 4.7) was added to the wells, which were further incubated overnight at 37°C . Optical density (OD) was measured with a microplate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA) at a wavelength of 562 nm. The OD of the NG cells was assigned a relative value of 100. The experiments were performed in triplicate.

Measurement of MCP-1 by ELISA

A piece of snap-frozen renal tissue and phenol-free media were used for the measurement of MCP-1. Renal tissue was pulverized with a mortar and pestle while frozen, homogenized in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, and a broad-spectrum proteinase inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Both renal tissue homogenates and conditioned media were centrifugation at 4,000 g for 20 min at 4°C , and the supernatants were collected and stored at -80°C .

The levels of MCP-1 were determined using a commercial ELISA kit (Biosource, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 50 μl of incubation buffer, 50 μl of standard diluent buffer, and 50 μl of sample were dispensed into a 96-well microplate coated with anti-rat MCP-1 antibody, and then 50 μl of biotinylated anti-MCP-1 solution was added to each well and incubated for 90 min at room temperature. After the wells were washed four times, 100 μl of streptavidin-horseradish peroxidase was added and then incubated for 30 min at room temperature. After another four washes, addition of 100 μl of stabilized chromogen was followed by 30-min incubation at room temperature and in the dark. Finally, 100 μl of stop solution was added and the OD was determined at 450 nm using an ELISA microtiter plate reader. The kit for rat MCP-1 was species specific and sensitive up to 750 pg/ml. All the concentrations of MCP-1 were normalized to the total protein amount.

Total RNA Extraction

Total RNA from renal cortical tissue was extracted as previously described (18). Briefly, 100 μl of RNA STAT-60 reagent (Tel-Test, Friendswood, TX) was added to the renal cortical tissues, which were lysed by freezing and thawing three times. Another 700 μl of RNA STAT-60 reagent was then added, and the mixture was vortexed and stored for 5 min at room temperature. Next, 160 μl of chloroform was added and the mixture was shaken vigorously for 30 s. After 3 min, the mixture was centrifuged at 12,000 g for 15 min at 4°C and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by adding 400 μl of isopropanol and then pelleted by centrifugation at 12,000 g for 30 min at 4°C . The RNA precipitate was washed with 70% ice-cold ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. RNA yield and quality were assessed based on spectrophotometric measurements at wavelengths of 260 and 280 nm.

Table 1. Animal data

	6-Wk				3-Mo			
	C ($n = 8$)	C+Col ($n = 8$)	DM ($n = 8$)	DM+Col ($n = 8$)	C ($n = 8$)	C+Col ($n = 8$)	DM ($n = 8$)	DM+Col ($n = 8$)
Body weight g	425 \pm 6	418 \pm 5	286 \pm 5*	295 \pm 6*	623 \pm 7	611 \pm 9	315 \pm 7*	327 \pm 5*
Body wt/kidney wt, %	0.65 \pm 0.03	0.62 \pm 0.05	1.12 \pm 0.11*	0.85 \pm 0.08	0.55 \pm 0.04	0.53 \pm 0.06	1.25 \pm 0.13†	0.83 \pm 0.06‡
Glucose, mg/dl	107 \pm 6	110 \pm 5	486 \pm 11†	475 \pm 15†	111 \pm 8	110 \pm 8	477 \pm 16†	465 \pm 14†
BUN, mg/dl	19.3 \pm 1.1	21.3 \pm 1.5	24.4 \pm 2.3	22.9 \pm 1.7	20.5 \pm 1.0	21.1 \pm 1.3	25.3 \pm 2.1	22.6 \pm 1.9
Creatinine, mg/dl	0.81 \pm 0.03	0.75 \pm 0.05	0.89 \pm 0.10	0.85 \pm 0.08	0.79 \pm 0.04	0.82 \pm 0.05	0.88 \pm 0.11	0.86 \pm 0.09
ALT, U/l	55 \pm 3	59 \pm 6	63 \pm 4	58 \pm 5	56 \pm 4	57 \pm 5	60 \pm 4	64 \pm 5
AST, U/l	115 \pm 9	110 \pm 13	109 \pm 11	118 \pm 13	111 \pm 10	113 \pm 9	121 \pm 11	115 \pm 12
24-h Urine albumin, mg/day	0.33 \pm 0.04	0.37 \pm 0.07	1.18 \pm 0.15*	0.57 \pm 0.13‡	0.38 \pm 0.06	0.35 \pm 0.07	2.89 \pm 0.20*	1.10 \pm 0.10‡

Values are means \pm SE. n = no. of rats; C, control; Col, colchicine; DM, diabetic; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase. * $P < 0.05$ vs. C and C+Col groups. † $P < 0.01$ vs. C and C+Col groups. ‡ $P < 0.05$ vs. DM group.

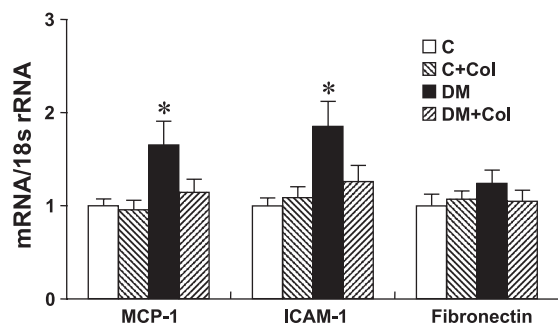


Fig. 1. Renal MCP-1, ICAM-1, and fibronectin mRNA/18s rRNA ratios in 6-wk control (C), C+colchicine (Col), diabetic (DM), and DM+Col rats. There were 1.7- and 1.9-fold increases in renal MCP-1/18s rRNA and ICAM-1/18s rRNA ratios, respectively, in DM compared with C rats, and these increases in MCP-1 and ICAM-1 mRNA expression were significantly abrogated by colchicine treatment. In contrast, there was no significant difference in the fibronectin mRNA/18s rRNA ratio among the 4 groups. * $P < 0.05$ vs. other groups.

Total RNA from mesangial cells and NRK-52E cells was extracted similarly.

Reverse Transcription

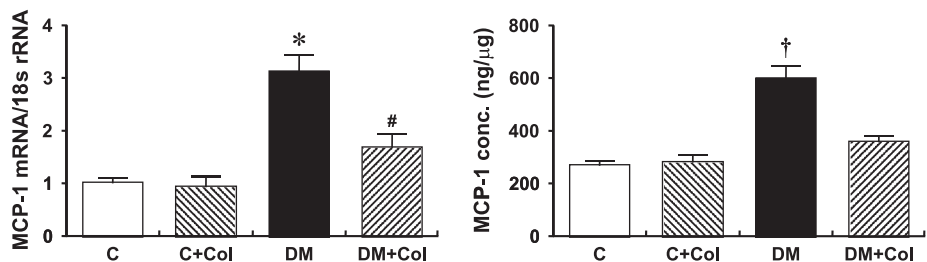
First-strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim). Two micrograms of total RNA extracted from renal cortex and cultured cells were reverse transcribed using 10 μ M random hexanucleotide primer, 1 mM dNTP, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreitol, 25 U RNase inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 min and 42°C for 1 h, followed by inactivation of the enzyme at 99°C for 5 min.

Real-Time PCR

The primers used for MCP-1, ICAM-1, fibronectin, and 18s amplification were as follows: MCP-1 sense 5'-TCTCTTCTCCACCACTATGCA-3', antisense 5'-GGCTGAGACAGCACGTGGAT-3'; ICAM-1 sense 5'-AGGTATCCATCCATCCAC-3', antisense 5'-GCCGAGGTCTCTGTTCTC-3'; fibronectin sense 5'-TGACAACCTGCCGTAGACCTGG-3', antisense 5'-TAC TGGTTGTAGGTGTGGCCG-3'; and 18s sense 5'-AGTCCCTGCCCTTTGT ACACA-3', antisense 5'-GATCCGAGGGCCCTCACTAAAC-3'. cDNAs from 25 ng RNA of renal cortical tissue or cultured cells per reaction tube were used for amplification.

Using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), PCR was performed with a total volume of 20 μ l in each well, containing 10 μ l of SYBR Green PCR Master Mix (Applied Biosystems), 5 μ l of cDNA, and 5 pM sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes to permit quantification of the gene normalized to the 18s rRNA. The PCR conditions were as follows: 35 cycles of denaturation at 94.5°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min.

Fig. 2. Renal MCP-1 mRNA/18s rRNA ratios and MCP-1 levels in 3-mo C, C+Col, DM, and DM+Col rats. There were 3.1- and 2.2-fold increases in the renal MCP-1/18s rRNA ratio and MCP-1 protein levels, respectively, in DM compared with C rats, and these increases in MCP-1 expression were significantly inhibited by the administration of colchicine. * $P < 0.01$ vs. C and C+Col. # $P < 0.05$ vs. DM. † $P < 0.05$ vs. other groups.



Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed for all PCRs.

After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. In addition, standard curves of threshold cycle values against the log values of a 10-fold serial dilution of cDNA were constructed and the amplification efficiencies were calculated for all target genes. The slopes of the standard curve for MCP-1, ICAM-1, fibronectin, and 18s were -3.36, -3.39, -3.45, and -3.28, respectively, and there was no significant difference in PCR efficiencies among the four genes. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta C_T}$. The results are given as relative expression of MCP-1, ICAM-1, and fibronectin normalized to the expression of the 18s housekeeping gene.

Western Blot Analysis

Renal cortical tissue and cultured cells harvested from plates were lysed in SDS sample buffer [2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol], treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in an 8% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in blocking buffer A (1 \times PBS, 0.1% Tween 20, and 8% nonfat milk) for 1 h at room temperature, followed by an overnight incubation at 4°C in a 1:1,000 dilution of polyclonal antibodies to rat ICAM-1 (R&D systems, Minneapolis, MN), fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), or β -actin (Sigma, St. Louis, MO). The membrane was then washed once for 15 min and twice for 5 min in 1 \times PBS with 0.1% Tween 20. Next, the membrane was incubated in buffer A containing a 1:1,000 dilution of horseradish peroxidase-linked donkey anti-goat IgG (Amersham Life Science, Arlington Heights, IL). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

Immunohistochemical Staining

For immunohistochemical staining, slices of kidney were snap-frozen in optimal cutting temperature (OCT) solution and 4- μ m sections of tissues were utilized. Slides were fixed in acetone for 10 min, air dried for 10 min at room temperature, and blocked with 10% donkey serum for 20 min at room temperature. For ICAM-1, fibronectin, and ED-1 staining, the primary polyclonal antibody to ICAM-1, the extracellular domain of fibronectin, or ED-1 (Chemicon International, Billerica, MA), respectively, was diluted 1:100 with 2% casein in BSA and applied for overnight incubation at room temperature. After washing, a secondary donkey anti-goat antibody was added for 20 min and the slides were then washed and incubated with a tertiary PAP complex for 20 min. DAB was added for 2 min, and the slides were counterstained with hematoxylin. To determine the semiquantitative score for ICAM-1 and fibronectin staining within the glomeruli and tubulointerstitial area, photographs of at least 20 glomeruli under

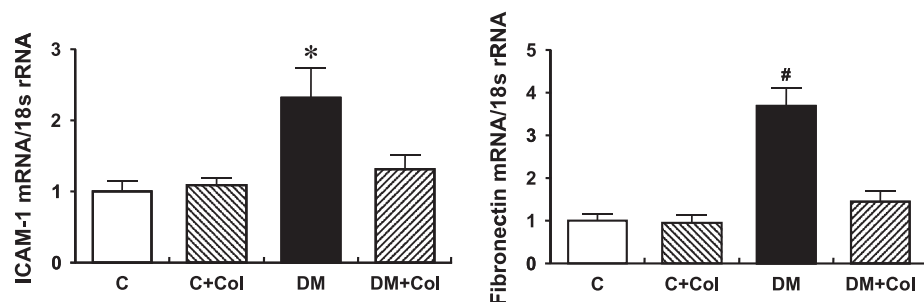


Fig. 3. Renal ICAM-1 and fibronectin mRNA/18s rRNA ratios in 3-mo C, C+Col, DM, and DM+Col rats. There were 2.3- and 3.7-fold increases in ICAM-1 and fibronectin mRNA/18s rRNA ratios, respectively, in DM relative to C rats, and these increases in ICAM-1 and fibronectin mRNA expression were significantly ameliorated with colchicine treatment. * $P < 0.05$ vs. other groups. # $P < 0.01$ vs. other groups.

$\times 400$ magnification and 20 tubulointerstitial fields under $\times 200$ magnification, respectively, were taken. Afterward, the positive-staining intensity of 1+ to 4+ compared with a negative control (score = 0), in which the immunohistochemical staining was performed without the primary antibodies, were defined by one investigator in a blinded fashion, based on the lightness and darkness of the brownish color using a digital image analyzer (MetaMorph version 4.6r5, Universal Imaging, Downingtown, PA). The staining score was obtained by multiplying the intensity of staining by the percentage of glomeruli or tubulointerstitium staining for that intensity; these numbers were then added for each experimental animal to give the staining score [$= \sum (\text{intensity of staining}) \times (\% \text{ of glomeruli or tubulointerstitium with that intensity})$]. The number of ED-1-positive cells was counted in at least 20 glomeruli and 20 fields of the tubulointerstitium/section under $\times 400$ magnification.

Determination of Podocyte Numbers

Immunohistochemical staining for WT-1 was also performed to determine the number of podocytes as previously described (2). Briefly, two adjacent semithin sections 3 μm apart were observed in pairs at a magnification of $\times 400$, and the nuclei present in the top section but not in the bottom section were counted and summed. Ten glomeruli in 5 rats from each group and 13–15 semithin sections from the midglomerular area were examined.

Statistical Analysis

All values are expressed as means \pm SE. Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Chicago, IL). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney U -test. The correlation between fibronectin mRNA expres-

sion and the number of ED-1-positive cells within the glomeruli and tubulointerstitium was determined by Pearson's correlation analysis. P values < 0.05 were considered to be statistically significant.

RESULTS

Animal Studies

Animal data. All animals gained weight over the experimental period, but body weight was highest in C rats. The ratios of kidney weight to body weight in DM rats at 6 wk and 3 mo were significantly higher than those in C and C+Col rats, and the increase in the ratio of kidney weight to body weight in 3-mo DM rats was significantly abrogated by the administration of colchicine ($P < 0.05$). The mean blood glucose levels in DM and DM+Col rats were significantly higher compared with C and C+Col rats throughout the study period ($P < 0.01$). Compared with the C and C+Col groups, 24-h urinary albumin excretion at 6 wk and 3 mo was significantly higher in DM rats, and colchicine treatment significantly reduced albuminuria in DM rats ($P < 0.05$). The serum levels of BUN and creatinine were slightly higher in DM rats compared with the other groups but did not reach statistical significance. On the other hand, there was no difference in ALT and AST levels among the four groups (Table 1).

In the preliminary experiments using diabetic rats treated with 2 U/day of insulin (Ultralente, Eli Lilly, Indianapolis, IN), the increase in MCP-1, ICAM-1, and fibronectin expression in 3-mo diabetic kidneys was significantly ameliorated by insulin treatment, suggesting that these changes in STZ-induced diabetic rats were not due to STZ per se. The results of this study,

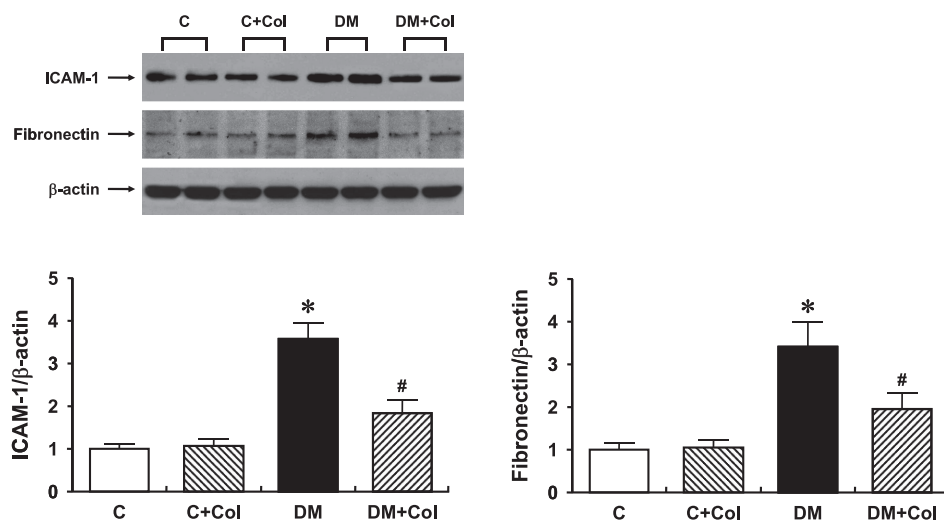


Fig. 4. Representative Western blot of ICAM-1 and fibronectin in 3-mo C, C+Col, DM, and DM+Col rats (representative of four blots). Renal ICAM-1 and fibronectin protein expression were significantly increased in DM compared with C rats, and these increases were significantly attenuated by the administration of colchicine. In contrast, there was no difference in β -actin protein expression among the four groups. * $P < 0.01$ vs. C and C+Col. # $P < 0.05$ vs. DM.

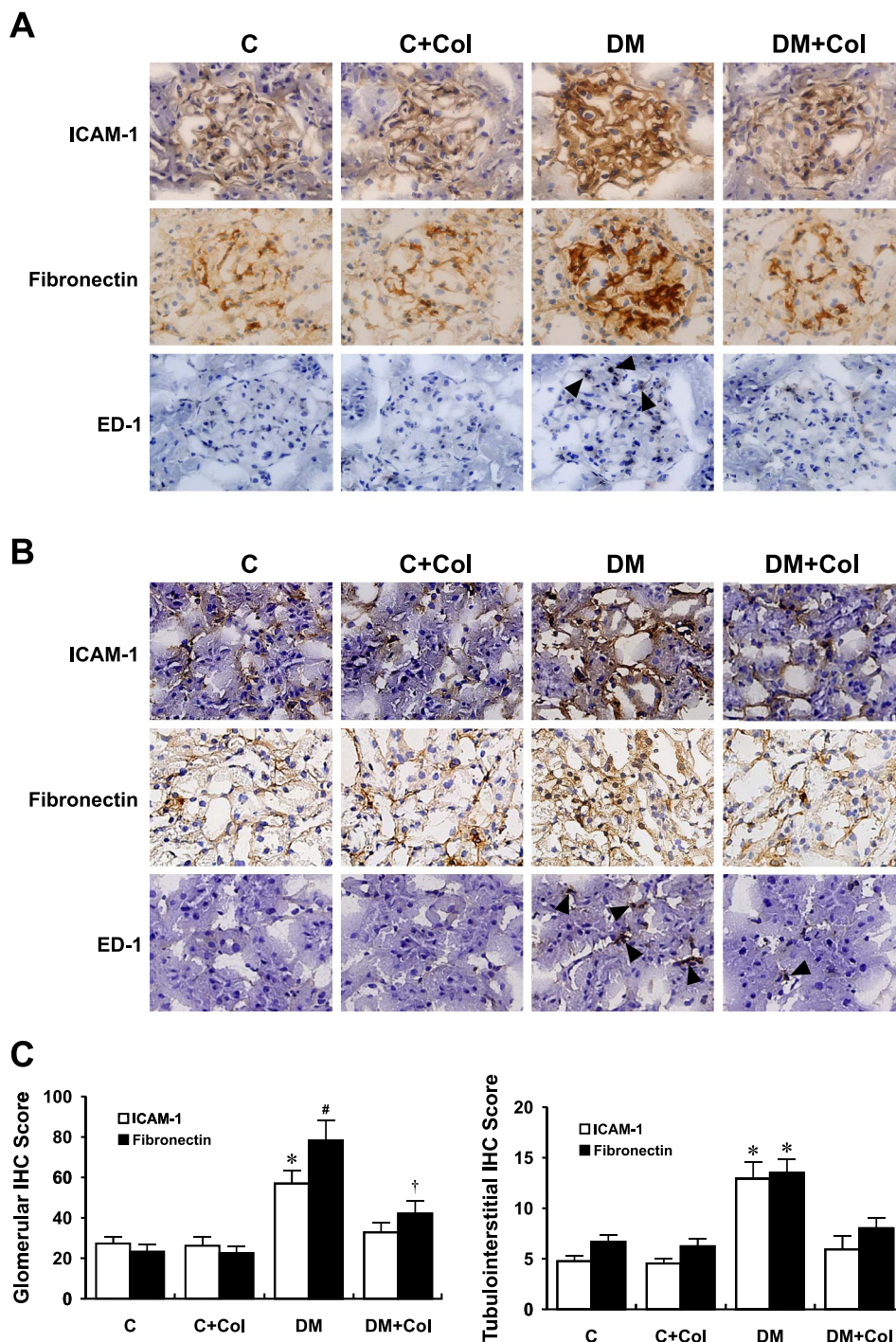
therefore, included only the data for C, C+Col, DM, and DM+Col rats.

Effect of colchicine on renal cortical MCP-1, ICAM-1, and fibronectin mRNA expression at 6 wk. Renal MCP-1 and ICAM-1 mRNA expression assessed by real-time PCR was significantly increased in DM compared with C rats ($P < 0.05$), and these increases were significantly abrogated by the administration of colchicine ($P < 0.05$). The MCP-1/18s rRNA and ICAM-1/18s rRNA ratios were 1.7- and 1.9-fold higher in DM compared with C rats, respectively, and colchicine treatment ameliorated these increases by 76.9 and 69.0%, respec-

tively. In contrast, there was no significant difference in fibronectin mRNA expression among the four groups (Fig. 1).

Effect of colchicine on renal cortical MCP-1 mRNA expression and protein levels at 3 mo. Renal MCP-1 mRNA expression was significantly increased in 3-mo DM compared with C rats ($P < 0.01$), and this increase in MCP-1 mRNA expression was significantly inhibited by the administration of colchicine ($P < 0.05$). The MCP-1 mRNA/18s rRNA ratio was 3.1-fold higher in 3-mo DM compared with C rats, and colchicine treatment attenuated this increase by 67.9% (Fig. 2). The levels of renal MCP-1 assessed by ELISA were also significantly

Fig. 5. Immunohistochemical staining (IHC) for ICAM-1, fibronectin, and ED-1 (as a marker of macrophages) in 3-mo C, C+Col, DM, and DM+Col rats. Glomerular (A) and tubulointerstitial (B) ICAM-1 and fibronectin staining were significantly increased in DM relative to C rats, and these increases were significantly abrogated by colchicine treatment. In addition, there were significantly more ED-1-positive cells (arrowhead) in DM compared with C rats, and colchicine significantly reduced the number of glomerular and tubulointerstitial macrophages in DM rats. C: IHC scores for ICAM-1 and fibronectin within the glomeruli and the tubulointerstitial area were significantly higher in DM rats relative to C rats, and colchicine treatment significantly inhibited these increases in DM rats ($\times 400$). * $P < 0.05$ vs. other groups. # $P < 0.01$ vs. C and C+Col. † $P < 0.05$ vs. DM.



higher in 3-mo DM relative to C rats (598.4 ± 49.3 vs. 268.3 ± 18.5 ng/ μ g, $P < 0.05$), and the increase in MCP-1 levels in DM rats was significantly ameliorated by colchicine treatment (357.3 ± 25.3 ng/ μ g, $P < 0.05$) (Fig. 2).

Effect of colchicine on renal cortical ICAM-1 and fibronectin mRNA expression at 3 mo. As seen in Fig. 3, ICAM-1 mRNA/18s rRNA ratio was significantly higher in 3-mo DM compared with C rats ($P < 0.05$), and colchicine treatment significantly attenuated this increase in renal ICAM-1 mRNA expression in DM rats ($P < 0.05$). To elucidate the effect of colchicine on ECM accumulation in experimental diabetic nephropathy, fibronectin expression was determined with renal cortical tissue. The renal fibronectin mRNA/18s rRNA ratio was significantly higher in 3-mo DM relative to C rats ($P < 0.01$), and this increase was significantly inhibited with colchicine treatment ($P < 0.01$) (Fig. 3).

Effect of colchicine on renal cortical ICAM-1 and fibronectin protein expression at 3 mo. Renal ICAM-1 and fibronectin protein expression assessed by Western blotting was also significantly increased in 3-mo DM compared with C rats ($P < 0.01$), and colchicine treatment significantly abrogated these increases in DM rats ($P < 0.05$) (Fig. 4). In addition, immunohistochemical staining for ICAM-1 and fibronectin confirmed the real-time PCR and Western blot findings. There were significant increases in glomerular and tubulointerstitial ICAM-1 and fibronectin staining in the 3-mo DM relative to the C group, and these increases in DM rats were significantly ameliorated by the administration of colchicine (Fig. 5).

Effect of colchicine on macrophage accumulation in diabetic nephropathy at 3 mo. The number of glomerular and tubulointerstitial macrophages (mainly located in the interstitium) assessed by immunohistochemical staining with ED-1 antibody was significantly higher in 3-mo DM compared with C rats (22.2 ± 4.2 vs. 3.4 ± 0.4 , $P < 0.005$), and colchicine treatment significantly decreased the number of ED-1-positive cells in DM rats (9.6 ± 1.0) ($P < 0.01$) (Fig. 5).

Correlation between fibronectin mRNA expression and the number of ED-1-positive cells. A correlation analysis was performed to elucidate the relationship between fibronectin mRNA expression and macrophage infiltration within the glomeruli and tubulointerstitium ($n = 32$). Fibronectin mRNA expression was significantly correlated with the number of infiltrated ED-1-positive cells in the kidney ($r = 0.81$, $P < 0.001$) (Fig. 6).

Podocyte numbers at 3 mo. Compared with C (168.3 ± 8.2) and C+Col rats (171.1 ± 6.7), the number of podocytes was significantly decreased in DM rats (139.9 ± 6.5), and the reduction in podocyte numbers was inhibited in DM rats treated with colchicine (162.1 ± 5.5) ($P < 0.05$).

Cell Culture Studies

Effect of colchicine on cell viability. The concentrations of colchicine (10^{-8} M) used in this study did not affect cell viability, which was assessed by an MTT assay ($97.3 \pm 8.2\%$ vs. NG).

Effect of colchicine on MCP-1 mRNA expression and protein levels. MCP-1 mRNA expression assessed by real-time PCR was significantly increased in HG-stimulated mesangial cells ($P < 0.01$) and NRK-52E cells ($P < 0.05$), and this increase in MCP-1 mRNA expression was significantly inhibited

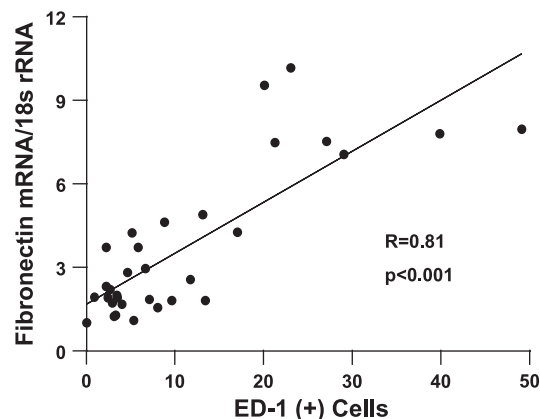


Fig. 6. Correlation between fibronectin mRNA expression and the number of ED-1-positive cells. Fibronectin mRNA expression correlated significantly with the number of infiltrated macrophages in the glomeruli and tubulointerstitium.

by colchicine treatment ($P < 0.05$). Compared with NG cells, the MCP-1 mRNA/18s rRNA ratios were 2.1- and 1.9-fold higher in mesangial cells and tubular epithelial cells exposed to HG, respectively, and colchicine treatment abrogated these increases by 75.3 and 84.7%, respectively (Fig. 7). The levels of MCP-1 protein in conditioned culture media assessed by ELISA showed a similar pattern to the mRNA expression (Fig. 7).

Effect of colchicine on ICAM-1 and fibronectin mRNA expression. High glucose significantly induced ICAM-1 and fibronectin mRNA expression in mesangial cells and NRK-52E cells. Compared with NG cells, the ICAM-1 mRNA/18s rRNA ratios were 2.2- and 2.1-fold higher in HG-stimulated mesangial cells and tubular epithelial cells ($P < 0.01$), respectively, and these increases were ameliorated by 85.4 and 80.2%, respectively, with colchicine treatment ($P < 0.05$) (Fig. 8). The fibronectin mRNA/18s rRNA ratio was also significantly increased in HG-stimulated mesangial cells ($P < 0.01$) and NRK-52E cells ($P < 0.05$) relative to NG cells by 132.3 and 74.0%, respectively, and these increases were significantly attenuated with colchicine treatment ($P < 0.05$) (Fig. 8).

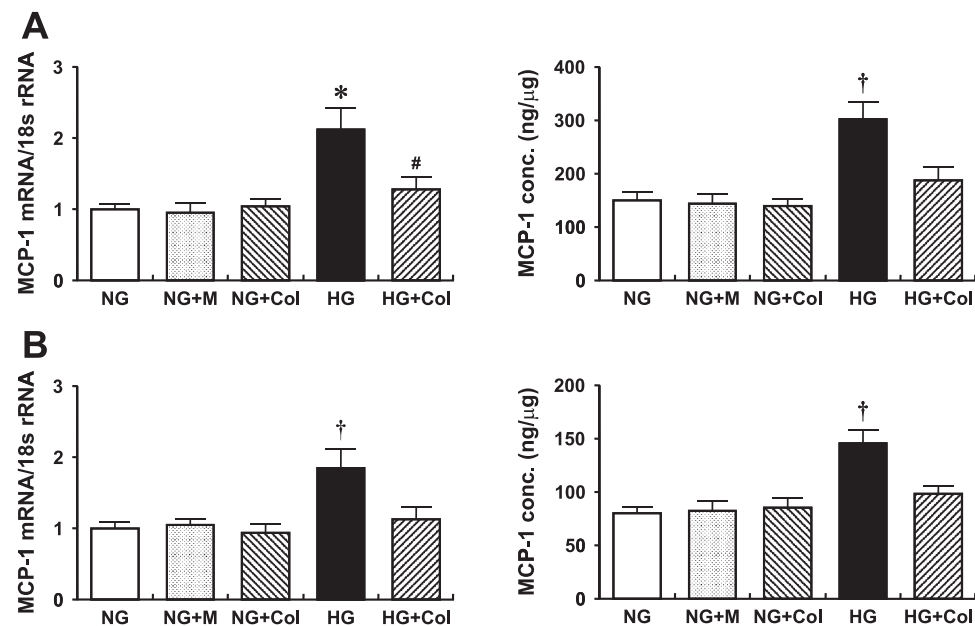
Effect of colchicine on ICAM-1 and fibronectin protein expression. ICAM-1 and fibronectin protein expression in cultured mesangial cells and NRK-52E cells showed similar patterns to their respective mRNA expression (Fig. 9).

DISCUSSION

Recent clinical and experimental studies have demonstrated that colchicine prevents renal injury in various glomerular and tubulointerstitial diseases (11, 24, 25, 27, 31, 32), but the effects of colchicine on proteinuria and pathological changes have never been explored in diabetic nephropathy. In this study, we demonstrate for the first time that inflammatory cell infiltration and fibronectin expression in diabetic glomeruli and tubulointerstitium are ameliorated by the administration of colchicine. In addition, the results of this study suggest that the anti-inflammatory effect of colchicine in diabetic nephropathy is partly mediated by inhibiting the increase in MCP-1 and ICAM-1 expression under diabetic conditions.

Colchicine is an alkaloid drug that has been used for many decades in acute gouty arthritis. Colchicine binds to tubulin

Fig. 7. MCP-1 mRNA/18s rRNA ratios and secreted MCP-1 protein levels in cultured mesangial cells (A) and NRK-52E cells (B) exposed to 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+ 10^{-8} M colchicine (NG+Col), 30 mM glucose (HG), or HG+ 10^{-8} M colchicine (HG+Col) medium. Compared with NG cells, the MCP-1 mRNA/18s rRNA ratios were 2.1- and 1.9-fold higher in mesangial cells and tubular epithelial cells exposed to HG, respectively, and colchicine treatment attenuated these increases by 75.3 and 84.7%, respectively. The levels of MCP-1 protein in conditioned culture media assessed by ELISA showed a similar pattern to the mRNA expression. * $P < 0.01$ vs. NG, NG+M, and NG+Col. # $P < 0.05$ vs. HG. † $P < 0.05$ vs. other groups.

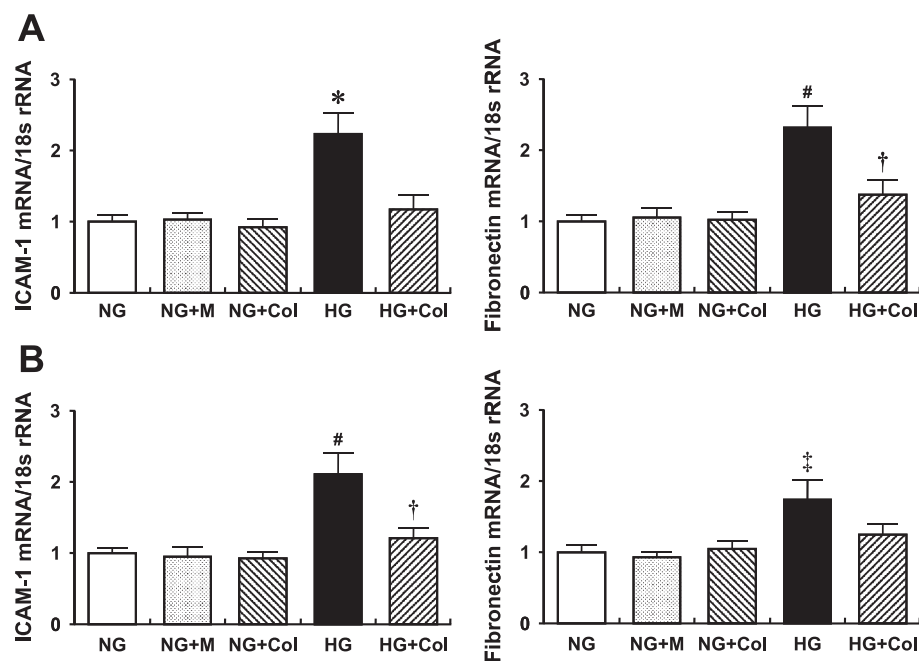


molecules and inhibits their polymerization into microtubules, resulting in disruption of the mitotic spindles (17). Due to this basic property, colchicine is mainly considered to be an anti-mitotic drug. However, accumulating evidence has shown that colchicine has additional effects on leukocytes and fibroblasts (9, 10, 15, 33, 37). Colchicine alters leukocyte functions such as adhesion and cytokine production (10). However, it has been suggested that the main anti-inflammatory effect of colchicine is attributed to its effect on leukocyte chemotaxis, which is known to occur even at a very low concentrations of colchicine (33). In addition, colchicine inhibits the release of fibronectin and collagen to the extracellular space, reduces collagen-processing enzyme, stimulates tissue collagenase activity, and inhibits the proliferation of fibroblasts (9, 10, 15). Based on

these anti-inflammatory and antifibrotic effects of colchicine, it has been used effectively to treat various diseases such as familial Mediterranean fever (13, 48), primary biliary cirrhosis (19, 23), and Behcet's syndrome (47).

The beneficial effects of colchicine have also been demonstrated in several experimental kidney disease models. McClurkin et al. (27) showed that colchicine treatment protected renal function and reduced fibrosis in a rabbit model of severe crescentic glomerulonephritis. They observed significantly lower serum creatinine concentrations and less renal interstitial fibrosis in colchicine-treated animals compared with the vehicle-treated group. In that study, however, regression analysis failed to identify the sites at which colchicine was acting. Improvement of interstitial fibrosis by colchicine was also

Fig. 8. ICAM-1 and fibronectin mRNA/18s rRNA ratios in cultured mesangial cells (A) and NRK-52E cells (B) exposed to 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+ 10^{-8} M colchicine (NG+Col), 30 mM glucose (HG), or HG+ 10^{-8} M colchicine (HG+Col) medium. Compared with NG cells, the ICAM-1 mRNA/18s rRNA ratios were 2.2- and 2.1-fold higher in HG-stimulated mesangial cells and tubular epithelial cells, respectively, and these increases were abrogated by 85.4 and 80.2%, respectively, with colchicine treatment. The fibronectin mRNA/18s rRNA ratio was also significantly increased in HG-stimulated mesangial cells and NRK-52E cells relative to NG cells by 132.3 and 74.0%, respectively, and these increases were significantly ameliorated with colchicine treatment. * $P < 0.01$ vs. other groups. # $P < 0.01$ vs. NG, NG+M, and NG+Col. † $P < 0.05$ vs. HG. ‡ $P < 0.05$ vs. other groups.



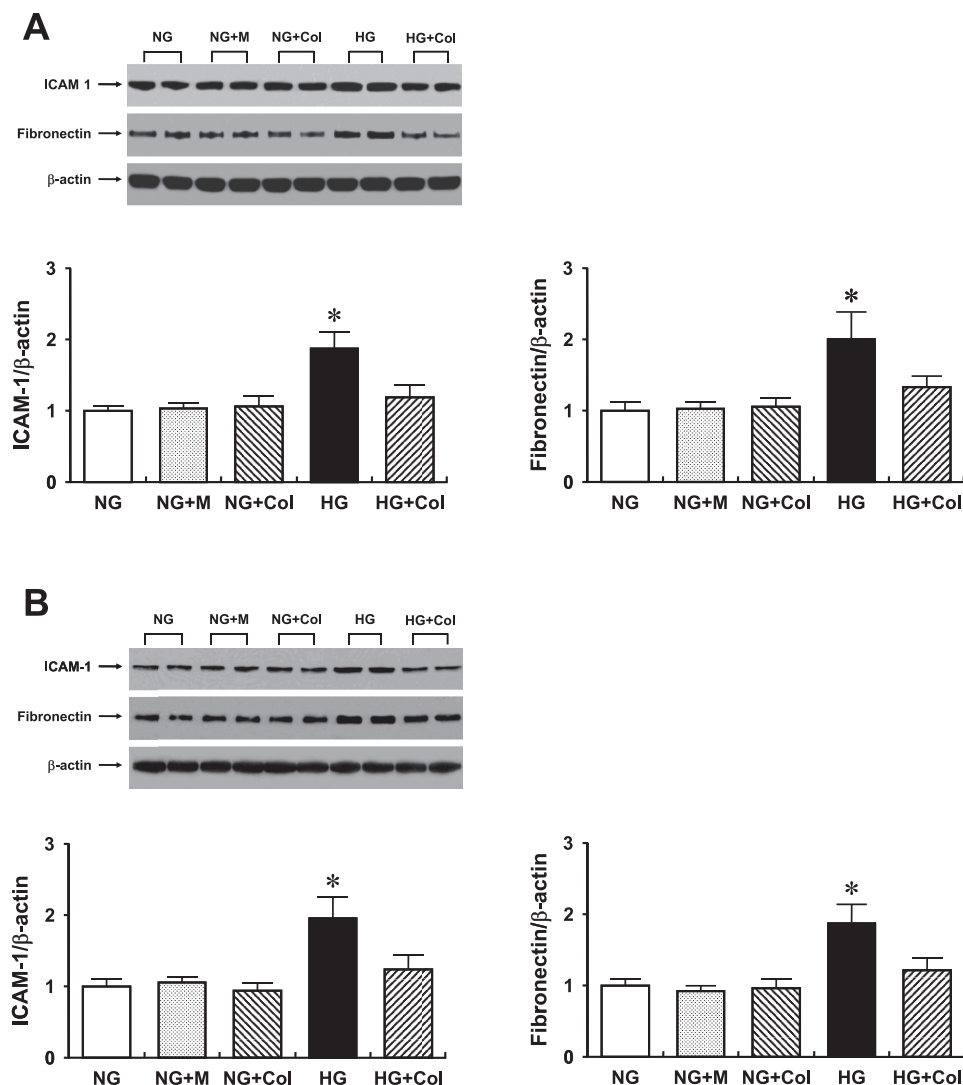


Fig. 9. Representative Western blot of ICAM-1 and fibronectin in cultured mesangial cells (A) and NRK-52E cells (B) (representative of 4 blots). Cellular ICAM-1 and fibronectin protein expression were significantly increased in HG-stimulated mesangial cells and tubular epithelial cells compared with NG cells, and these increases were significantly attenuated by colchicine treatment. In contrast, there was no difference in β -actin protein expression among the groups. * $P < 0.05$ vs. other groups.

demonstrated in an animal model of chronic cyclosporine nephrotoxicity (11, 24, 25). A recent study demonstrated that cyclosporine-induced histological changes and the increases in transforming growth factor (TGF)- β expression, apoptotic cells, and serum malonyldialdehyde levels were inhibited by the administration of colchicine (11), suggesting that the protective effects of colchicine for cyclosporine nephrotoxicity were attributed to the reduction in TGF- β overexpression, apoptosis, and oxidative damage. In contrast to the uniform antifibrotic effect of colchicine, the effect of colchicine on inflammatory cell infiltration is not consistent. Some studies demonstrated that there were no significant differences in inflammatory cell infiltration between colchicine- and vehicle-treated rats with chronic cyclosporine nephrotoxicity (41), whereas one study showed that colchicine treatment significantly decreased the number of ED-1-positive cells along with the decreases in osteopontin mRNA and protein expression in these rats (24). The reasons for these differences in the anti-inflammatory effect of colchicine in cyclosporine nephrotoxicity are not clear, but differences between species, duration of the animal experiments, and kidney lesions observed to determine the number of infiltrated inflammatory cells may contribute

to these disparities. The results of our study reveal decreases in glomerular and tubulointerstitial fibronectin accumulation and inflammatory cells infiltration in colchicine-treated diabetic rats, suggesting that the antifibrotic and anti-inflammatory effects of colchicine may also be operative in diabetic nephropathy.

Even though the diabetic milieu per se, hemodynamic changes, and local growth factors are considered mediators in the pathogenesis of diabetic nephropathy, recent studies suggest that an inflammatory mechanism may also contribute to the development of diabetic nephropathy and that MCP-1 and ICAM-1 are considered the central molecules involved in macrophage/monocyte influx in diabetic nephropathy (5–7, 30, 42). In this study, we observed that MCP-1 and ICAM-1 expression was increased in experimental diabetic nephropathy, which was associated with inflammatory cell infiltration, and in high glucose-stimulated mesangial cells and tubular epithelial cells, and these increases under diabetic conditions were attenuated by colchicine treatment. Taken together, the anti-inflammatory effect of colchicine in diabetic nephropathy can be partly attributed to the suppression of MCP-1 and ICAM-1 expression in mesangial cells and tubular epithelial

cells by colchicine. Recently, several studies have demonstrated that podocytes express MCP-1 and its expression is enhanced by high glucose and advanced glycation end-products (14, 16), suggesting that podocytes are also involved in the inflammatory process under diabetic conditions. In addition, administration of MMF and cartilage oligomeric matrix protein-angiopoietin-1 suppressed MCP-1 and prevented podocyte loss and foot process effacement in type 1 and type 2 diabetic animals, respectively (22, 49). Since 24-h urinary albumin excretion and podocyte loss were abrogated in diabetic rats by the administration of colchicine in this study, we surmise that colchicine may improve podocyte changes in diabetic rats, but further study will be needed to clarify the direct effect of colchicine on podocytes under diabetic conditions.

Once recruited monocytes/macrophages are activated, they release lysosomal enzymes, nitric oxide, reactive oxygen species, platelet-derived growth factor (PDGF), tumor necrosis factor- α , interleukin (IL)-1, and TGF- β , and in turn promote renal injury (29, 35, 46). PDGF stimulates fibroblast proliferation (20, 44), and IL-1 induces the expression of TGF- β , the most well-known profibrotic cytokine, in fibroblasts (45). In MCP-1- and ICAM-1-deficient diabetic mice, renal fibrosis also was significantly reduced along with less inflammatory cell infiltration (5–7, 30), suggesting that the inhibition of inflammatory cell recruitment may lead to a reduction in extracellular matrix accumulation. The results of the present study also showed that the increase in fibronectin expression came after the increases in MCP-1 and ICAM-1 expression. Therefore, we argue that one of the interesting findings of this study, the amelioration of glomerular and tubulointerstitial ECM accumulation in colchicine-treated diabetic rats, is a consequence of reduced infiltration of inflammatory cells in addition to the aforementioned antifibrotic effect of colchicine.

In summary, colchicine ameliorates inflammatory cell infiltration in diabetic nephropathy by inhibiting MCP-1 and ICAM-1 expression. In addition, enhanced fibronectin expression in the diabetic kidney is attenuated by colchicine treatment. These findings suggest that colchicine could be a useful drug for preventing nephropathy in diabetes.

ACKNOWLEDGMENTS

Present address of D. K. Kim: Dept. of Internal Medicine, Seoul National University Hospital, 28 Yongon-Dong, Chongno-Gu, Seoul, Korea 110-744.

GRANTS

This work was supported by the BK21 (Brain Korea 21) Project for Medical Sciences, Yonsei University, and a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (MOST; R01-2007-000-20263-0 and R13-2002-054-04001-0).

REFERENCES

- Banba N, Nakamura T, Matsumura M, Kuroda H, Hattori Y, Kasai K. Possible relationship of monocyte chemoattractant protein-1 with diabetic nephropathy. *Kidney Int* 58: 684–690, 2000.
- Basgen JM, Nicholas SB, Mauer M, Rozen S, Nyengaard JR. Comparison of methods for counting cells in the mouse glomerulus. *Nephron Exp Nephrol* 103: 139–148, 2006.
- Chiarelli F, Cipollone F, Mohn A, Marini M, Iezzi A, Fazia M, Tumini S, De Cesare D, Pomilio M, Pierdomenico SD, Di Gioacchino M, Cuccurullo F, Mezzetti A. Circulating monocyte chemoattractant protein-1, and early development of nephropathy in type 1 diabetes. *Diabetes Care* 25: 1829–1834, 2002.
- Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state, and progressive renal injury. *Kidney Int* 65: 116–128, 2004.
- Chow FY, Nikolic-Paterson DJ, Ma FY, Ozols E, Rollins BJ, Tesch GH. Monocyte chemoattractant protein-1-induced tissue inflammation is critical for the development of renal injury but not type 2 diabetes in obese db/db mice. *Diabetologia* 50: 471–480, 2007.
- Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Rollins BJ, Tesch GH. Monocyte chemoattractant protein-1 promotes the development of diabetic renal injury in streptozotocin-treated mice. *Kidney Int* 69: 73–80, 2006.
- Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Tesch GH. Interleukin-1 deficiency is protective against nephropathy in type 2 diabetic db/db mice. *J Am Soc Nephrol* 16: 1711–1722, 2005.
- Coimbra TM, Janssen U, Grone HJ, Ostendorf T, Kunter U, Schmidt H, Brabant G, Floege J. Early events leading to renal injury in obese Zucker (fatty) rats with type I.I. diabetes. *Kidney Int* 57: 167–182, 2000.
- Dehm P, Prockop DJ. Time lag in the secretion of collagen by matrix-free tendon cells, and inhibition of the secretory process by colchicine and vinblastine. *Biochim Biophys Acta* 264: 375–382, 1972.
- Diegelmann RF, Peterkofsky B. Inhibition of collagen secretion from bone, and cultured fibroblasts by microtubular disruptive drugs. *Proc Natl Acad Sci USA* 69: 892–896, 1972.
- Disel U, Paydas S, Dogan A, Gulfiliz G, Yavuz S. Effect of colchicine on cyclosporine nephrotoxicity, reduction of TGF-beta overexpression, apoptosis, and oxidative damage: an experimental animal study. *Transplant Proc* 36: 1372–1376, 2004.
- Furuta T, Saito T, Ootaka T, Soma J, Obara K, Abe K, Yoshinaga K. The role of macrophages in diabetic glomerulosclerosis. *Am J Kidney Dis* 21: 480–485, 1993.
- Goldfinger SE. Colchicine for familial Mediterranean fever. *N Engl J Med* 287: 1302, 1972.
- Gu L, Hagiwara S, Fan Q, Tanimoto M, Kobata M, Yamashita M, Nishitani T, Gohda T, Ni Z, Qian J, Horikoshi S, Tomino Y. Role of receptor for advanced glycation end-products, and signalling events in advanced glycation end-product-induced monocyte chemoattractant protein-1 expression in differentiated mouse podocytes. *Nephrol Dial Transplant* 21: 299–313, 2006.
- Harris ED Jr, Krane SM. Effects of colchicine on collagenase in cultures of rheumatoid synovium. *Arthritis Rheum* 14: 669–684, 1971.
- Han SY, So GA, Jee YH, Han KH, Kang YS, Kim HK, Kang SW, Han DS, Han JY, Cha DR. Effect of retinoic acid in experimental diabetic nephropathy. *Immunol Cell Biol* 82: 568–576, 2004.
- Hastie SB. Interactions of colchicine with tubulin. *Pharmacol Ther* 51: 377–401, 1991.
- Kang SW, Adler SG, Nast CC, LaPage J, Gu JL, Nadler JL, Natarajan R. 12-lipoxygenase is increased in glucose-stimulated mesangial cells, and in experimental diabetic nephropathy. *Kidney Int* 59: 1354–1362, 2001.
- Kaplan MM, Alling DW, Zimmerman HJ, Wolfe HJ, Sepersky RA, Hirsch GS, Elta GH, Glick KA, Eagen KA. A prospective trial of colchicine for primary biliary cirrhosis. *N Engl J Med* 315: 1448–1454, 1986.
- Knecht A, Fine LG, Kleinman KS, Rodemann HP, Müller GA, Woo DD, Norman JT. Fibroblasts of rabbit kidney in culture. II. Paracrine stimulation of papillary fibroblasts by PDGF. *Am J Physiol Renal Physiol* 261: F292–F299, 1991.
- Ledwozyw A. The effect of colchicine, and vinblastine on bleomycin-induced lung fibrosis in rats. *Acta Physiol Hung* 82: 383–389, 1994.
- Lee S, Kim W, Moon SO, Sung MJ, Kim DH, Kang KP, Jang KY, Lee SY, Park BH, Koh GY, Park SK. Renoprotective effect of COMP-angiopoietin-1 in db/db mice with type 2 diabetes. *Nephrol Dial Transplant* 22: 396–408, 2007.
- Lee YM, Kaplan MM. Efficacy of colchicine in patients with primary biliary cirrhosis poorly responsive to ursodiol, and methotrexate. *Am J Gastroenterol* 98: 205–208, 2003.
- Li C, Yang CW, Ahn HJ, Kim WY, Park CW, Park JH, Cha JH, Kim J, Kim YS, Bang BK. Colchicine suppresses osteopontin expression, and inflammatory cell infiltration in chronic cyclosporine nephrotoxicity. *Nephron* 92: 422–430, 2002.
- Li C, Yang CW, Ahn HJ, Kim WY, Park CW, Park JH, Lee MJ, Yang JH, Kim YS, Bang BK. Colchicine decreases apoptotic cell death in chronic cyclosporine nephrotoxicity. *J Lab Clin Med* 139: 364–371, 2002.
- Liu CY, Gu ZL, Zhou WX, Guo CY. Effect of Astragalus complanatus flavonoid on anti-liver fibrosis in rats. *World J Gastroenterol* 11: 5782–5786, 2005.
- McClurkin C Jr, Phan SH, Hsu CH, Patel SR, Spicker JK, Kshirsagar AM, Yuan WY, Wiggins RC. Moderate protection of renal function, and

- reduction of fibrosis by colchicine in a model of anti-GBM disease in the rabbit. *J Am Soc Nephrol* 1: 257–265, 1990.
28. **Morii T, Fujita H, Narita T, Shimotomai T, Fujishima H, Yoshioka N, Imai H, Kakei M, Ito S.** Association of monocyte chemoattractant protein-1 with renal tubular damage in diabetic nephropathy. *J Diabetes Complications* 17: 11–15, 2003.
29. **Nikolic-Paterson DJ, Atkins RC.** The role of macrophages in glomerulonephritis. *Nephrol Dial Transplant* 16, Suppl 5: 3–7, 2001.
30. **Okada S, Shikata K, Matsuda M, Ogawa D, Usui H, Kido Y, Nagase R, Wada J, Shikata Y, Makino H.** Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes. *Diabetes* 52: 2586–2593, 2003.
31. **Oner A, Erdogan O, Demircin G, Bulbul M, Memis L.** Efficacy of colchicine therapy in amyloid nephropathy of familial Mediterranean fever. *Pediatr Nephrol* 18: 521–526, 2003.
32. **Ozdemir BH, Ozdemir FN, Sezer S, Sar A, Haberal M.** Does colchicine have an antifibrotic effect on development of interstitial fibrosis in renal allografts of recipients with familial Mediterranean fever? *Transplant Proc* 38: 473–476, 2006.
33. **Phelps P.** Polymorphonuclear leukocyte activity in vitro. IV. Colchicine inhibition of chemotactic activity formation after phagocytosis of urate crystals. *Arthritis Rheum* 13: 1–9, 1970.
34. **Prodjosudjadi W, Gerritsma JS, Klar-Mohamad N, Gerritsen AF, Bruijn JA, Daha MR, van Es LA.** Production, and cytokine-mediated regulation of monocyte chemoattractant protein-1 by human proximal tubular epithelial cells. *Kidney Int* 48: 1477–1486, 1995.
35. **Rehan A, Johnson KJ, Wiggins RC, Kunkel RG, Ward PA.** Evidence for the role of oxygen radicals in acute nephrotoxic nephritis. *Lab Invest* 51: 396–402, 1984.
36. **Rennard SI, Bitterman PB, Ozaki T, Rom WN, Crystal RG.** Colchicine suppresses the release of fibroblast growth factors from alveolar macrophages in vitro. The basis of a possible therapeutic approach of the fibrotic disorders. *Am Rev Respir Dis* 137: 181–185, 1998.
37. **Rinehart JJ, Boulware T.** Microfilament, and microtubule function in human monocytes. *J Lab Clin Med* 90: 737–743, 1977.
38. **Rodríguez L, Cerbón-Ambriz J, Muñoz ML.** Effects of colchicine, and colchicine in a biochemical model of liver injury and fibrosis. *Arch Med Res* 29: 109–116, 1998.
39. **Rovin BH, Yoshimura T, Tan L.** Cytokine-induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. *J Immunol* 148: 2148–2153, 1992.
40. **Sassy-Prigent C, Heudes D, Mandet C, Belair MF, Michel O, Perdreau B, Bariety J, Bruneval P.** Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. *Diabetes* 49: 466–475, 2000.
41. **Sobh M, Sabry A, Moustafa F, Foda MA, Sally S, Ghoneim M.** Effect of colchicine on chronic cyclosporin nephrotoxicity in Sprague-Dawley rats. *Nephron* 79: 452–457, 1998.
42. **Sugimoto H, Shikata K, Hirata K, Akiyama K, Matsuda M, Kushiro M, Shikata Y, Miyatake N, Miyasaka M, Makino H.** Increased expression of intercellular adhesion molecule-1 (ICAM-1) in diabetic rat glomeruli: glomerular hyperfiltration is a potential mechanism of ICAM-1 upregulation. *Diabetes* 46: 2075–2081, 1997.
43. **Utamura R, Fujihara CK, Mattar AL, Malheiros DM, Noronha IL, Zatz R.** Mycophenolate mofetil prevents the development of glomerular injury in experimental diabetes. *Kidney Int* 63: 209–216, 2003.
44. **Van Zoelen EJ, Van Rotterdam W, Van de Wetering RA, Heldin CH.** Differential effects of PDGF isoforms on proliferation of normal rat kidney cells. *Growth Factors* 9: 329–339, 1993.
45. **Vesey DA, Cheung C, Cuttle L, Endre Z, Gobe G, Johnson DW.** Interleukin-1 β stimulates human renal fibroblast proliferation, and matrix protein production by means of a transforming growth factor- β -dependent mechanism. *J Lab Clin Med* 140: 342–350, 2002.
46. **Young BA, Johnson RJ, Alpers CE, Eng E, Gordon K, Floege J, Couser WG.** Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47: 935–944, 1995.
47. **Yurdakul S, Mat C, Tuzun Y, Ozyazgan Y, Hamuryudan V, Uysal O, Senocak M, Yazici H.** A double-blind trial of colchicine in Behcet's syndrome. *Arthritis Rheum* 44: 2686–2692, 2001.
48. **Zemer D, Revach M, Pras M, Modan B, Schor S, Sohar E, Gafni J.** A controlled trial of colchicine in preventing attacks of familial Mediterranean fever. *N Engl J Med* 291: 932–934, 1974.
49. **Zhang Y, Chen B, Hou XH, Guan GJ, Liu G, Liu HY, Li XG.** Effects of mycophenolate mofetil, valsartan, and their combined therapy on preventing podocyte loss in early stage of diabetic nephropathy in rats. *Chin Med J* 120: 988–995, 2007.